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FOREWORD

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Zesheng Liu, Ph.D. 6/30/04

PI - Signature Date

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Abstract

Tumor growth and metastasis is a complex pathophysiological process comprising of various interactions between tumor cells and the host(1-3). A significant approach in understanding tumor invasion and metastasis is by identifying the genes involved in tumor growth and suppression, and investigating their molecular mechanisms and eventually in their role as therapeutic agent, if any. Maspin is a tumor suppressor gene related to the serine protease inhibitor (serpin) family(4). It is expressed in normal prostate epithelial cells, but is down regulated as normal prostate cells progress from a pre-neoplastic to an invasive state. This down regulation is mediated *in vitro* at the transcriptional level through cis-acting elements and trans-acting factors in the maspin promoter(5)[i]. Further, recombinant maspin is shown to inhibit prostate tumor-induced growth and neovascularization in a xenograft nude mouse model and inhibit tumor invasion and motility of prostatic cell lines *in vitro(6)*.

This proposal aims to investigate the tumor suppressor function of maspin *in vivo,* and thereby as a novel molecular target in the treatment of prostate cancers. The specific aims for this two-year proposal are:

Specific Aim 1. To investigate the effect of maspin in a syngeneic xenograft mouse model of prostate cancer.

Specific Aim 2. To establish a transgenic mouse overexpressing maspin in the prostate to study the role of gain of function of maspin in prostate cancer.

Objective 1: To generate a bitransgenic mice expressing maspin in the prostate in the context of an inducible system

Objective 2: To cross the bigenic maspin mice with TRAMP mice to investigate the tumor suppressor function of maspin *in vivo*.

In the last two years, both specific aims were initiated. We have tested the effect of maspin in a syngeneic xenograft mouse model of prostate cancer. Maspin cDNA was introduced into TRAMP prostate tumor C2N cells. Parental C2N cells and maspin stable cells were injected subcutaneously into C57BL/6 mice. We show that tumor growth was blocked in maspin expressing tumors in vivo. Further analyses show that maspin inhibits angiogenesis and induces tumor cell apoptosis. These results have been summarized for the preparation of manuscript. With regard to the second specific aim, we have generated floxed beta-geo-maspin transgenic mice. These mice have also been crossed with PBARR2-cre mice to generate bitransgenic mice. Unfortunately, two transgenic mouse lines we generated all expressed very low level of maspin transgene after Cre recombianse deletion. As with all animal experiments, this problem is hard to predict. We are trying alternative approaches to overcome the problem at the moment.

Body

Materials and methods

Animals

Syngeneic C57BL/6 mice (for implantation of TRAMP tumor cells) were purchased from Harlan, Inc. All animals were maintained within the PI's animal facility at Baylor.
TRAMP cell lines were obtained from Dr. Norm Greenberg at Baylor College of Medicine.

Antibodies

Polyclonal anti-maspin antibody was made by Zymed, Inc. as a custom service. All secondary antibodies were purchased from Zymed, Inc.

Northern and Western analysis

RNAs and proteins were isolated from cells, prostate tissues. Total RNAs were isolated using Gibco/BRL Trizol regent. For northern blot, roughly 20 ug RNA will be loaded each lane. For Western blot analysis, protein extracts were prepared by lysing the cells in non-ionic detergent-containing buffers. Total 100 ug protein extract will be loaded for electrophoresis.

Immunohistochemical analysis

Prostate tissues were removed from male mice and dissected. Tissues were fixed in 10% neutral formalin buffer and embedded in paraffin and sectioned at 5 μm. For maspin immunostaining, tissues were boiled in citrate buffer (Zymed, Inc.) for ten minutes for antigen retrival. The antibody was produced in rabbit against a fifteen amino acid peptide located in the reactive site loop of maspin, a region designated as AbS4A. The antibody was purified using an AbS4A sulfo-linked affinity column (Sulfolink kit, Pierce, IL). The sections were stained with the affinity purified maspin antibody at a dilution of 1:400, followed by a secondary goat anti-rabbit antibody staining, and the color was developed by Zymed's AEC (3-Amino-9-Ethylcarbazole) chromogen kit. For specific peptide blocking, a concentration of 10 nM of AbS4A peptide was preincubated with antibody for thirty minutes at room temperature. For proliferating cell nuclear antigen (PCNA) staining, a PCNA staining kit was purchased from Zymed (Zymed, Inc., CA) and slides were stained following the instruction of the kit.

Results and Discussion

Task 1. Examination of maspin as a tumor suppressor in prostate.

We have overexpressed maspin in TRAMP (TRansgenic Animal Model of Prostate cancer) prostate tumor cells by retrovirus infection(7). Retroviral stable transfectants were selected. We used a C2N mouse prostate tumor cell line that was initially isolated from TRAMP tumors in Dr. Norman Greenburg's laboratory. Parental C2N cells and maspin stable cells were injected subcutaneously into C57BL/6 mice. Almost all of C2N cells inoculated developed into palpable tumors. However, none of the maspin stable clones have developed any

Table 1. Effect of maspin overexpression on prostate tumor development

		C2N-46	C2N	
Group 1,2	% of tumor development	0 % (0 /25)	90.5% (19/21)	P<0.001
	Tumor free period (days)	180	54.61±10.92	
	Tumor observation (days)		57.06±12.34	
	% of lung metastasis		42.1% (8/19)	

tumors after extended time of observation, demonstrating those maspin functions to inhibit prostate tumor in vivo (Table 1, Fig.1). When both C2N and C2N-maspin clones were cultured in vitro, both cells grew rapidly in the presence of 5% fetal bovine serum medium. In 3D spheroid culture, both cells grew into similar size of spheres. We collected the condition medium from C2N-maspin cells and carried out western blot analysis with maspin antibody. Our data showed that C2N-maspin medium contained detectable maspin protein. We compared the tumor cell apoptosis rate between C2N and C2N-maspin cells. The maspin expressing C2N-Maspin tumor cells had significant increase of apoptosis than that of C2N control cells (Fig.2). In addition, we carried out experiments to determine whether the secreted maspin from C2N-maspin tumors could inhibit tumor growth by affecting angiogenesis. Using the collected conditional medium we showed that endothelial cell migration was significantly inhibited (Fig.3). Thus, the reason for the tumor inhibition in maspin clones in vivo may be due to the combined effect of increased tumor cell apoptosis and inhibition of angiogenesis.

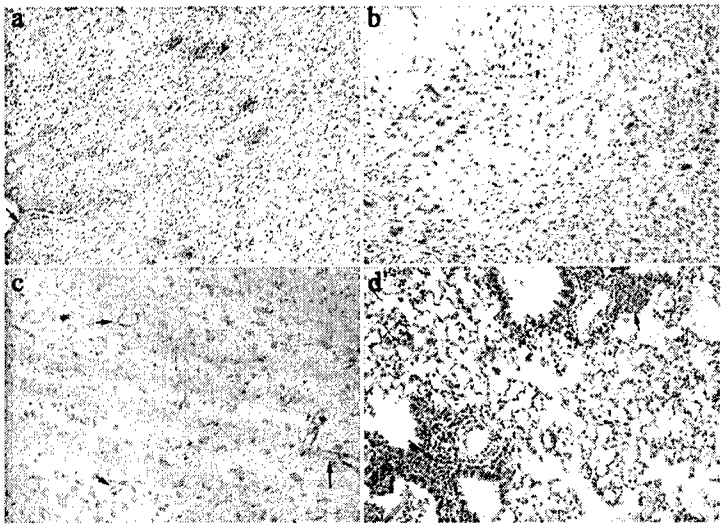
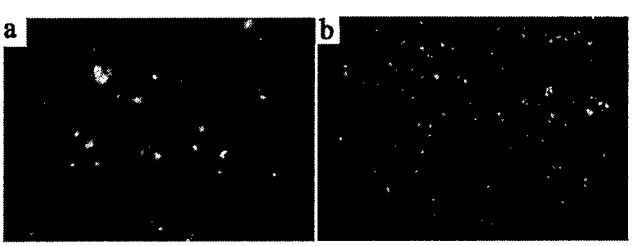


Fig.1. Analysis of C2N prostate primary tumor, tumor vessel density, and lung metastasis by histology and immunohistochemistry. a. Histology of C2N tumor by H&E. Note tumor cells invade to the skin keratinocytes (arrow). B. Lack of tumor encapsulation in C2N tumor section. c. Immunohistolochemistry of CD31. Arrows indicate the tumor vessels. d. Histology of lung metastasis stained by H&E. Arrows indicate tumor cells.



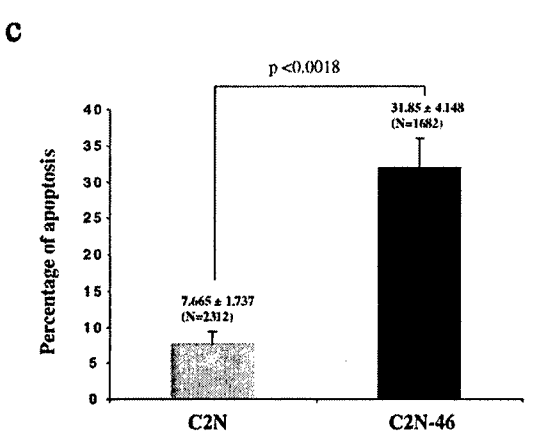


Fig.2. TUNEL analysis of C2N and C2N-46 tumor spheroids. TUNEL staining of apoptotic cells in C2N (a) and C2N-46 (b) spheroids. Green cells indicated TUNEL positive cells. All cells were stained by DAPI (blue). c. Summary of TUNEL analysis for C2N and C2N-46 spheroids. Total number of cells counted were indicated as the N.

Task 2. To generate a bitransgenic mice expressing maspin in the prostate in the context of an inducible system

We have made a DNA construct to target maspin overexpression in prostate. This construct uses a actin promoter to direct beta-geo gene expression. This beta-geo is floxed by LoxP sites (locus of X-over of P1, which is the 34-bp on the P1 genome recognized by bacteriophage P1Cre protein (cyclization recombination)). Mouse maspin is ligated to the

construct following the LoxP sites. In the absence of cre recombinase, only beta-geo is expressed. However, when cre is targeted by a prostate specific promoter, such as the androgen responsive region (ARR2) promoter (developed by Bob Matusik), maspin will be turned on in prostate tissue. Major progresses have been made in the generation of such tissue specific transgenic mice. Firstly, we have made the DNA construct and injected the construct for the generation of transgenic mice. The transgenic mice were born and preliminary data showed that beta-geo was expressed in the tissue of prostate. Secondly, through a collaboration with Dr. Feng Wang at Texas A& M University, we have obtained a strain of ARR2-cre mice. This strain has been crossed with ROSA26 reporter line to demonstrate the prostate specific expression of cre recombinase. ROSA26 reporter line is a transgenic mouse strain that upon cre cleavage expresses beta-galactosidase (beta-gal). We have breed our floxed beta-geo-maspin transgenic mice with Dr. Wang's ARR2-cre to generate bitransgenic mice. Unfortunately, two parental transgenic mouse lines we generated all expressed very low level of maspin transgene after Cre recombianse deletion. Although the B-geo signal is present we believe that chick-b-actin promoter that was used to direct the beta-geo/maspin expression is too weak in prostate. We are taking two approaches to overcome the difficult in making the tissue specific maspin transgenic mice. The first is to inject more old construct to generate more transgenic lines so that we have the possibility to select one high maspin expression line after crossing with Arr2-cre. The other possibility is to change the chicken actin promoter into a elongation factor promoter. This promoter is capable of driving gene expression in prostate tissue (personal communication with Dr. Alexander, BCM). We are now in the process of generating more transgenic lines for the screening and will proceed with the second approach if our first approach fails. I would like to emphasize that we have done the experiments as proposed in the statement of work in the last two years, although the result was not what we expected. Despite the fact that this fellowship has already expired, we remain hopeful to continue to build a transgenic mouse model for prostate study. Thus, we will continue the experiments to study the effect of maspin overexpression on prostate development and tumorigenesis.

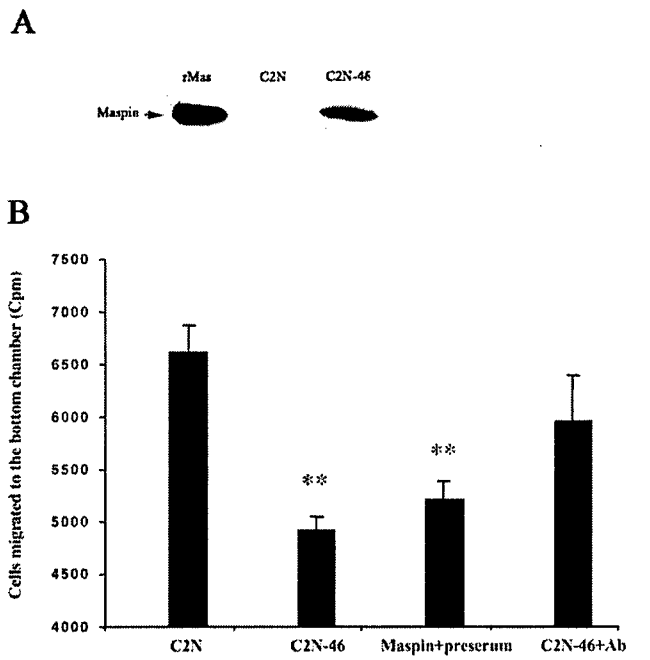


Fig.3. Secretion of maspin by C2N-46 and inhibition of endothelial cell migration. a. Western blot analysis of maspin using condition medium collected from C2N and C2N-46. Bacterial recombinant maspin protein (rMas) was used as control. Condition medium was concentrated 20X fold. b. The effect of maspin on HUVEC endothelial cell migration. HUVEC cells were treated by condition medium from C2N, C2N-46, C2N-46 plus preserum, C2N-46 plus maspin antibody. Note condition medium of C2N-46 inhibited HUVEC cell migration and this effect was maspin specific. ** indicates statistical significance compared to C2N control ($p < 0.003$).

Key research accomplishments

Clones of prostate tumor cells without (C2N) and with (C2NM) maspin expression grew equally well in liquid and semisolid culture media, but only the C2N cells developed into palpable tumors in C57BL/6 mice. Condition medium from C2N-maspin cells were collected for western blot analysis with maspin antibody. Our data showed that C2N-maspin medium contained detectable level of maspin protein. Using the collected conditional medium we showed that endothelial cell migration was significantly inhibited. These data indicate that maspin prevents tumor angiogenesis in mice. We are currently preparing a manuscript for this study. Transgenic mice of B-geo/maspin have been established and have been crossed with ARR2-Cre transgenic mice. However, due to some technical problems, the level of maspin remains too low. We are currently building more mouse lines to select for better transgenic strain.

Reportable outcome

Products

1. Establishing a syngeneic tumor implantation model using C2N TRAMP tumor cells

2. Animal models developed

- Transgenic mice were developed to express maspin and the beta-geo marker.
- Bitransgenic mice were developed to express the ROSA26 reporter and thereby demonstrate expression of cre recombinase by ARR2-cre mice.

We are currently focusing to study the effect of maspin overexpression on prostate development and the transgenic mice will be crossed with prostate oncogenic mice to examine the role of maspin tumorigenesis.

Conclusion

Two tasks proposed in the grant were initiated in the first year of proposal. Key reagents have been obtained and transgenic mice have been developed. Continuation of the tasks in the next few years will help us understand the role of maspin in prostate development and tumor metastasis, and hopefully leading to the development of new therapies for the treatment of prostate cancer.

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